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EFFECT OF PHARMACOLOGIC AGENTS ON HUMAN KERATINOCYTE MITOSIS IN VITRO

III. Inhibition by Histamine and Methylated Analogs

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Keratinocytes from normal human skin were propagated in vitro. Histamine at 2×10^{-6} M inhibited mitosis 68%. The side-chain methylated analogs, *n*-methylhistamine and *n,n*-dimethylhistamine, produced an inhibitory response of 53 and 60%, respectively, at 1×10^{-6} M. The side-chain acetylated analog, *n*-acetylhistamine, did not elicit an inhibitory response in concentrations as high as 1×10^{-4} M. The ring-methylated analogs, 1-methylhistamine and 4-methylhistamine, produced some degree of mitotic inhibition at 1×10^{-4} M. When the H_1-blocking agent, pyrilamine, or the H_2-blocking agent, metiamide, was added in conjunction with histamine, the histamine-induced mitotic inhibition was abolished. These data indicate that human keratinocytes may possess receptors for histamine which could play a role in the regulation of human keratinocyte proliferation in vivo.

For decades histamine has been known to occur in the skin of mammals, mainly being located in mast cells of the dermis [1]. Much evidence indicates that histamine is a vasodilator involved in the pathogenesis of *normal* inflammatory responses and urticaria [2,3]. While a specific function for histamine in the physiologic behavior of epidermis has not been established, it was reported by Voorhees et al [4] that histamine in high concentrations (3.5×10^{-2} M) stimulated rat epidermal mitoses in vitro. Data were presented to support the hypothesis that the increased mitoses were due to a decrease in the concentration of intracellular cyclic AMP caused by histamine stimulation of the soluble epidermal cyclic AMP phosphodiesterase. These same authors later reported that histamine stimulation of rat epidermal mitosis may not be related to decreased cyclic AMP levels, but rather to increased synthesis of cyclic GMP [5].

In an earlier paper, we reported a contrary observation, namely that histamine inhibited the flow of human keratinocytes in vitro through the G_2 part of the cell cycle [6]. We have now verified that observation and, in addition, have shown that (1) neither precursor molecule nor breakdown products exhibit activity at comparable concentrations of histamine, (2) the methylated side-chain derivatives retain full activity while the ring-methylated analogs do not, and (3) the block in G_2 may be mediated by histamine receptors.

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MATERIALS AND METHODS

Normal human skin was obtained at surgery and primary outgrowth cultures of epidermal keratinocytes were made from explants according to a method described elsewhere [7]. Each explant gives rise to an outgrowing sheet of epithelial cells whose origin from epidermal keratinocytes has previously been demonstrated [7,8]. The cells are propagated in Eagle's minimum essential medium containing 10% fetal calf serum plus 100 U/ml each of penicillin, streptomycin, and mycostatin. After 7 to 10 days in culture approximately 200,000 cells are present in each dish. Cultures are maintained at 37°C in a high-humidity incubator in a mixture of 5% CO_2 in air.

To study the effect of histamine and related compounds on mitosis, a 4-hr in vitro metaphase collection assay was employed. Each compound was dissolved in culture fluid and an appropriate aliquot, never more than 1% of the total volume, was added to each dish to achieve the desired concentration. The metaphase arrest agent, Colcemid (3.5 μg/ml) was added along with each test compound. After 4 hr the cultures were fixed in 10% buffered formalin, the explants removed from the coverslips, and the outgrowths stained in acid hematoxylin. Controls containing only Colcemid were run with each experiment. To determine the mitotic index (M.I.), the entire number of arrested metaphases is counted in each outgrowth. The total number of cells in each outgrowth is determined by the method of Chopra [9]. The M.I. is expressed as the number of arrested metaphases per 1000 cells. Since the assay covers only a 4-hr period and G_2 is known to be at least 7 hr in this system [10], any effect on the M.I. is probably due to inhibition in G_2. For each dish the M.I. was obtained for each outgrowth and then the mean M.I. and standard deviation were calculated. The

statistical significance of the data obtained for each experiment was evaluated by using Student's *t*-test for unpaired data. The data in the Tables represent one experiment for each concentration listed. However, for each compound tested at the various concentrations, two or more experiments were carried out using skin obtained from different patients. Due to normal fluctuations in donor skin metabolism, control values vary from experiment to experiment. However, the final results (% inhibition) were consistent within the experimental error of the biologic assay. To study the effect of histamine-blocking agents, the blocking agent was added to the culture ½ hr before addition of the amine and Colcemid for the usual 4 hr.

Histamine dihydrochloride was purchased from Sigma Chemical Co. (St. Louis, Mo.). Pyrilamine and metiamide were gifts from Dr. Marshall Guthrie (Smith, Kline and French Laboratories, Philadelphia, Pa.); *n*-methylhistamine, *n,n*-dimethylhistamine, and 4-methylhistamine were kindly supplied to us by Dr. W. A. M. Duncan (Smith, Kline and French Laboratories, England); and 1-methylhistamine and *n*-acetylhistamine were purchased from Calbiochem. (San Diego, Cal.).

RESULTS

At concentrations ranging from 2×10^{-2} to 2×10^{-6} M, histamine substantially inhibited the progression of cells through G_2 (Tab. I). The maximum inhibition that could be obtained was 68% at 2×10^{-6} M. The level of inhibition dropped to zero at 2×10^{-8} M. Various imidazole-related compounds had no effect on inhibiting mitosis at concentrations comparable to that of histamine (Tab. II). Imidazole itself did inhibit mitosis a 1×10^{-3} M, but this activity dropped to zero at 1×10^{-4} M.

The side-chain methyl derivatives, *n*-methylhistamine and *n,n*-dimethylhistamine, produced mitotic inhibition comparable to histamine (Tab. III). It would appear that the two side-chain derivatives produce comparable inhibition of cells in G_2. The acetylated side-chain analog, *n*-acetylhistamine, produced no mitotic inhibition even at concentrations as high as 1×10^{-4} M. The ring-methylated derivatives exhibit some degree of activity of 1×10^{-4} M; however, this decreases to a residual level at 1×10^{-5} M.

At a concentration of 1×10^{-6} M, the H_1-blocking agent, pyrilamine, and the H_2-blocking agent, metiamide, exhibited no mitotic inhibition. When either of these blocking agents was added in conjunction with histamine, the histamine-induced inhibition was effectively abolished (Tab. IV).

TABLE I. *Effect of histamine on human keratinocyte mitosis in vitro*[a]

Histamine Conc (M)	M.I.[b] ± SD		Percent Inhibition
	Control	Test	
2×10^{-2}	19.5 ± 5.2	6.5 ± 2.6 ($p < 0.001$)	67
2×10^{-5}	20.2 ± 2.7	7.4 ± 0.8 ($p < 0.001$)	63
2×10^{-6}	20.2 ± 2.7	6.4 ± 3.5 ($p < 0.001$)	68
2×10^{-7}	20.2 ± 2.7	14.7 ± 1.1 ($p < 0.005$)	27
2×10^{-8}	20.2 ± 2.7	22.8 ± 4.0 ($p > 0.150$)	0 (+12)

[a] For normal skin p values <0.05 are considered to be statistically significant.

[b] M.I. (mitotic index) is the proportion of arrested metaphases per 1000 cells accumulated during the 4-hr period following Colcemid treatment.

DISCUSSION

There was a striking difference in response to histamine between the rat epidermal in vitro system and our human keratinocyte in vitro system. As mentioned previously, in rat epidermis, histamine *stimulates* mitosis either by acting on the cyclic AMP or cyclic GMP system [4,5]. However, in our human cell system, histamine exhibited strong *inhibition* of mitosis in concentrations from 2×10^{-2} M to 2×10^{-6} M. The reason for this "species" difference may be related to the experimental models employed. We are measuring responses of human keratinocytes propagated in vitro, whereas Voorhees et al [4] used "whole rat skin" in organ culture. The dermis in the latter system may be influencing the epidermal response to histamine. A species variation in the molecular structure of the receptor could also explain the different responses. It seems quite probable that histamine in our cell system is acting via the adenyl cyclase system [11–15]. More specifically, in human skin, histamine has been found to raise the level of cyclic AMP (K. Halprin, personal communication). Based on our previous studies [16] an increase in intracellular cyclic AMP would inhibit the flow of cells through G_2. However, the possibility that cyclic GMP levels are modulated by this amine cannot be overlooked.

We were very much interested in determining whether compounds containing the imidazole moiety could elicit a mitotic inhibitory response.

TABLE II. *Effect of imidazole compounds on human keratinocyte mitosis in vitro*

Compound	Conc (M)	M.I. ± SD		Percent Inhibition
		Control	Test	
Histidine	1×10^{-3}	21.9 ± 2.3	17.2 ± 3.1 ($p < 0.025$)	21
Urocanic acid	1×10^{-3}	33.5 ± 2.5	30.8 ± 1.3 ($p < 0.050$)	8
Imidazole acetate	1×10^{-3}	25.8 ± 1.0	20.0 ± 2.4 ($p < 0.010$)	22
	1×10^{-4}	25.8 ± 1.0	25.5 ± 1.4 ($p > 0.350$)	0
Imidazole	1×10^{-3}	25.8 ± 1.0	15.2 ± 1.5 ($p < 0.001$)	41
	1×10^{-4}	25.8 ± 1.0	25.2 ± 3.4 ($p > 0.350$)	0

TABLE III. *Effect of histamine analogs on human keratinocyte mitosis in vitro*

Amine	Conc (M)	M.I. ± SD		Percent Inhibition
		Control	Test	
n-Methylhistamine	1×10^{-4}	37.0 ± 4.6	6.7 ± 0.8 ($p < 0.001$)	82
	1×10^{-5}	37.0 ± 4.6	14.2 ± 1.6 ($p < 0.001$)	62
	1×10^{-6}	23.8 ± 0.3	11.2 ± 1.4 ($p < 0.001$)	53
	1×10^{-7}	21.5 ± 0.7	21.6 ± 1.6 ($p > 0.400$)	0
n,n-Dimethylhistamine	1×10^{-4}	37.0 ± 4.6	12.4 ± 2.9 ($p < 0.001$)	66
	1×10^{-5}	37.0 ± 4.6	15.9 ± 1.0 ($p < 0.001$)	57
	1×10^{-6}	23.8 ± 0.3	9.6 ± 1.0 ($p < 0.001$)	60
	1×10^{-7}	21.5 ± 0.7	20.7 ± 1.0 ($p > 0.100$)	4
n-Acetylhistamine	1×10^{-4}	37.0 ± 4.6	41.2 ± 7.5 ($p > 0.150$)	0 (+11)
1-Methylhistamine	1×10^{-4}	35.8 ± 0.4	22.6 ± 1.8 ($p < 0.001$)	37
	1×10^{-5}	21.5 ± 0.7	18.6 ± 2.9 ($p > 0.050$)	14
4-Methylhistamine	1×10^{-4}	35.8 ± 0.4	21.5 ± 1.7 ($p < 0.001$)	40
	1×10^{-5}	23.8 ± 0.3	20.3 ± 2.6 ($p < 0.050$)	15

TABLE IV. *Effect of H_1- and H_2-blocking agents on histamine-induced mitotic inhibition in human keratinocytes in vitro*

Compound	Conc (M)	M.I. ± SD		Percent Inhibition
		Control	Test	
Histamine	2×10^{-6}	23.6 ± 2.4	12.5 ± 1.0 ($p < 0.001$)	47
Pyrilamine[a]	1×10^{-6}	23.6 ± 2.4	23.4 ± 2.9 ($p > 0.450$)	0
Metiamide	1×10^{-6}	23.6 ± 2.4	21.4 ± 2.0 ($p > 0.100$)	9
Histamine + Pyrilamine	2×10^{-6} + 1×10^{-6}	23.6 ± 2.4	22.1 ± 2.1 ($p > 0.200$)	6
Histamine + Metiamide	2×10^{-6} + 1×10^{-6}	23.6 ± 2.4	19.7 ± 4.2 ($p > 0.050$)	16

[a] Blocking agents were added ½ hr before histamine.

This was especially true since histidine metabolism is very active in the epidermis. Our results showed that neither histidine, the precursor to histamine, nor one of its breakdown products, imidazole acetate, affected our cells. Urocanic acid which is normally found in large quantities in the epidermis [17] had no effect on mitosis. Imidazole did have a suppressive effect at 1×10^{-3} M. Since imidazole can stimulate cyclic AMP phosphodiesterase activity in vitro [18] leading to a decrease in cyclic AMP, such an effect should lead to an increase in mitosis, not a decrease. The explanation for the observed effect remains unclear.

It is important to note the activities of the various methylated analogs of histamine. Our data would support the conclusion that side-chain methylation preserves the activity of the histamine molecule. This is compatible with the in vivo data in other mammalian tissues which suggest that the products of side-chain methylation are as active or even more active than histamine [14]. On the other hand, acetylation of the side-chain terminal nitrogen abolished all activity. It would be conceivable that in skin in vivo the amount of histamine available to the epidermis could be controlled conveniently by acetylation. And indeed, one of the breakdown products of histamine in man is *n*-acetylhistamine. It would be of interest to see whether human skin, particularly the epidermis, has any of the necessary enzymes to degrade or inactivate histamine, and if any of these enzymes are elevated in hyperproliferative skin diseases such as psoriasis.

It was of some surprise to find evidence that human keratinocytes, at least in vitro, may have specific receptor sites for histamine. Since pyrilamine and metiamide blocked the action of histamine, no clear-cut distinction between H_1 and H_2

receptors can be made at this time. Studies are under way to differentiate the histamine receptor from the β-adrenergic receptor, since we reported earlier that human keratinocytes in vitro may contain β-receptors [19]. The existence of specific histamine receptor sites on keratinocytes suggests that histamine may play a regulatory role in control of epidermal mitosis.

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